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The culture of HaCaT cells on liquid substrates is mediated by a mechanically strong liquid-liquid interface

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The mechanical properties of naturally-derived matrices and biomaterials are thought to play an important role in directing cell adhesion, spreading, motility, proliferation and differentiation. However, recent reports have indicated that cells may respond to local nanoscale physical cues, rather than bulk mechanical properties. We had previously reported that primary keratinocytes and mesenchymal stem cells did not seem to respond to the bulk mechanical properties of poly(dimethyl siloxane) (PDMS) substrates. In this study, we examine the mechanical properties of weakly crosslinked PDMS substrates and observe a liquid-like behaviour, with complete stress relaxation. We then report the observation that HaCaT cells, an epidermal cell line, proliferate readily at the surface of uncrosslinked liquid PDMS, as well as a low viscosity (0.77 cSt) fluorinated oil. These results are suprising, considering the current views in the field of mechanotransduction on the importance of bulk mechanical properties, but we find that strong mechanical interfaces, presumably resulting from protein assembly, are formed at liquid-liquid interfaces for which cell adhesion and proliferation are observed. Hence our results suggest that cells sense the nanoscale mechanical properties of liquid-liquid interfaces and that such physical cues are sufficient to sustain the proliferation of adherent cells.

Cell adhesion, spreading, proliferation and differentiation are regulated by a complex collection of factors, including growth factors, cytokines, extracellular matrix composition and the presence of neighbouring cells[1,2]. In addition to biochemical cues, physical properties of this microenvironment have been shown to modulate cell phenotype[3,4]. For example the nanoscale topography of biomaterials was found to regulate the adhesion, spreading and fate of stem cells[5-7]. Similarly, mechanical properties of the ECM have been shown to affect cell adhesion, spreading[8,9], motility[10,11], proliferation[12,13], differentiation[14,15] and stem cell self-renewal[16]. Changes in ECM mechanics are also often correlated with pathologies such as cancer, where they are thought to contribute to the development of an abnormal and potentially invasive phenotype [17-19]. Such interactions not only have important implications for our understanding of biological processes and signalling underlying the control of cell phenotype, but also for the design of biomaterials supporting the long term expansion of stem cells as well as acting as scaffolds for the regeneration of damaged or diseased tissues.

In many cases, stem cell phenotype has been correlated with the bulk mechanical properties of substrates designed to study mechanostransduction, such as ECM protein-functionalised poly(acrylamide) or poly(ethylene glycol) hydrogels[14,15]. In the vast majority of these works, cells are most sensitive to stiffness in the range of 0.1-100 kPa, a very broad range considering it refers to forces that are thought to be generated via one single main contractile machinery (e.g. mediated by integrin, focal adhesion and the actin cytoskeleton). However, we previously reported that primary keratinocytes and mesenchymal stem cells (MSCs) did not apparently respond to the mechanical properties of hydrophobic substrates (crosslinked poly(dimethyl siloxane), PDMS): their spreading and differentiation remained unchanged on PDMS matrices with a range of Young's moduli of 0.1 kPa to 2 MPa[20]. This suggested that cells respond to nanoscale mechanical properties of their microenvironment, at least at the single cell level, rather than bulk mechanical properties. More recently, a number of other reports also evidenced that direct correlations between bulk mechanical properties and cell phenotypes are perhaps misleading. For example, whilst MSCs displayed maximum spreading and differentiated into osteoblasts on stiff poly(acrylamide) hydrogels[14], their spreading remained unchanged on soft quasi-2D nanofibrous mats compared to stiffer mats[21]. This was attributed to the increased remodelling of soft fibres and their clustering into bundles supporting the formation of focal adhesions. Similarly, whilst MSCs encapsulated into stiff 3D covalently crosslinked alginate hydrogels committed to osteogenic lineages[22], they differentiated into osteoblasts when seeded within soft viscoelastic alginate gels or degradable hyaluronic acid matrices[23]. In such cases, it was again the ability of cells to remodel their softer environment and allow the clustering of ligands that was attributed to the seemingly contradicting phenotypes observed.

These results indicate that cells sense other physical cues, in addition to bulk mechanics, such as nanoscale ligand clustering and matrix remodelling, and that these cues can dominate. This concept has already been proposed by Sheetz and Ladoux, who proposed that mechanosensing arises from nanoscale (near 100 nm) strain sensing events[24-26]. Recently, del Rio et al. confirmed the occurrence of such nanoscale strain sensing process by studying the spreading of cells on self-assembled monolayers displaying tethers of varying length. Single molecule force spectroscopy identified that a critical ligand displacement exists (falling between 40 and 200 nm) beyond which

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integrin receptors presumably disengage and that prevents cell spreading[27].

However, the absence of cell response we previously reported on PDMS substrates[20] was not fully explained and the precise origin of the divergence between bulk and nanoscale mechanical properties in such substrates remained unclear. Could such divergence arise from the rates at which cells probe their mechanical environment, compared to the rates at which mechanical characterisation is typically carried out? If not, what is the molecular origin of this divergence and can we quantify the corresponding nanoscale and bulk mechanical properties? Our work in this area led us to investigate further the mechanical properties of very weakly crosslinked Sylgard 184 PDMS substrates and to study the behaviour of cells spreading at the surface of non-crosslinked oils.

A. Materials and Methods

1. Materials

Trimethylamine, triethoxy(octyl)silane, trichloro (1H, 1H, 2H, 2H-perfluorooctyl) silane, 2,3,4,5,6-pentafluorobenzoyl chloride, poly(dimethyl siloxane) (PDMS, trimethyl siloxane terminated) were purchased from Sigma-Aldrich. The fluorinated oil (Novec 7500) was from ACOTA. Ethanol was purchased from VWR chemicals. DMEM cell culture medium, Hoechst 33342, LIVE/DEAD assay kit and versene were from Thermofisher Scientific. SYLGARD 184 PDMS was from Ellsworth. Reagents were used as received unless specified otherwise. Double distilled water (ddH2O) was obtained using a Purelab® Chorus system.

2. Formation of liquid-liquid interfaces for cell culture

24 well plates were plasma oxidized using a plasma coater (Diener, 100 % intensity) for 10 min. 500 µL ethanol, 10 µL trimethylamine and 10 µL of the desired silane (triethoxy(octyl)silane to prepare interfaces with liquid PDMS or trichloro (1H, 1H, 2H, 2H-perfluorooctyl) silane for the fluorinated oil) were added into each well. Ethanol was added in between wells to slow down evaporation and parafilm was used to seal the well plate cover. After incubating for 24 h, the wells were washed in a sterile environment with 70% ethanol (twice) and ddH2O (three times). 500 µL fluorinated oil with fluorinated surfactant (2,3,4,5,6-pentafluorobenzoyl chloride) at final concentrations of 0.01, 0.005 and 0 mg/mL was added in the fluorophilic 24 well plate to form the bottom liquid layer. Cell culture medium (2 mL, containing foetal bovine serum) was directly added into each well to generate corresponding liquid-liquid interfaces and cell were added shortly after the formation of these interfaces.

3. HaCaT culture and seeding

Human keratinocyte HaCaT cells were cultured in DMEM containing 10 % foetal bovine serum (FBS, Labtech), 1 % L-Glutamine (200 mM) and 1 % Penicillin-Streptomycin (5,000 U/mL). For proliferation assays, HaCaT cells were harvested with trypsin (0.25 %) and versene solutions (0.2 g/L EDTA in Phosphate Buffered Saline) in a ratio of 1/9, centrifuged, counted and resuspended in DMEM at the desired density before seeding onto substrates (conditioned as stated above, in a 24-well plate) at a density of 2,000 cells per well (1,000 cells per cm^2). Cells were left to adhere and proliferate in an incubator (37 °C and 5 % CO_2) for different periods of time, prior to staining and imaging. For passaging, cells were reseeded in a T75 at a density of 250k cells per flask.

3. Immunofluorescence staining and antibodies.

Immunofluorescence staining and antibodies. After washing once with PBS, samples were fixed with 4 % paraformaldehyde (Sigma-Aldrich) for 10 min and permeabilized with 0.2 % Triton X-100 (Sigma-Aldrich) for 5 min at room temperature. Following blocking for 1 h in 10 % foetal bovine serum and 0.25 % gelatine (from cold water fish skin, Sigma-Aldrich), substrates were incubated with primary antibodies for 1 h at room temperature, and with Alexa Fluor 488-conjugated secondary antibody (Thermofisher Scientific) for 1 h at room temperature (1:1000). When relevant, tetramethyl rhodamine isothiocyanate phalloidin (1:500, Sigma-Aldrich) was included in the blocking solution and DAPI （1:1000, Sigma-Aldrich） in the secondary antibody solution. Samples of cells adhering to oils were imaged directly without mounting.

4. Hoechst staining and LIVE/DEAD cell viability assay

Cell proliferation was assessed via Hoechst (nuclear) staining. Cells were incubated in DMEM containing 5 µL Hoechst 33342 (1 mg/mL stock solution) for 30 min before imaging by epifluorescence microscopy (see below). Viability of HaCaT cells on fluorinated oil interfaces was quantified by LIVE/DEAD viability/cytotoxicity assay using a kit. In brief, HaCaT cells were incubated in DMEM with 2 µM Calcein AM and 4 µM Ethidium homodimer for 30 min. stained cells were imaged using a Leica DMI4000 fluorescence microscopy (see below). The percentage of viable cells was calculated by counting the number of green (live) cells and dividing by the total number of cells.

5. Fluorescence microscopy and data analysis

Fluorescence microscopy images were acquired with a Leica DMI4000B fluorescence microscope (CTR4000 lamp; 63 × 1.25 NA, oil lens; 10 × 0.3 NA lens; 2.5 x 0.07 NA lens; DFC300FX camera). To determine cell densities (per area), cell counting was carried out by thresholding and watershedding nuclear images in ImageJ. In the case of cell clumps, for which this protocol did not allow the isolation of individual nuclei, cells were counted manually. Statistical analysis was carried out using Origin 8 through one-way ANOVA with Tukey test for posthoc analysis. Significance was determined by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and n.s., non-significant.

6. Rheology of cured PDMS samples

Sylgard 184 PDMS samples were prepared at a base/cross linker ratio of 100/1. The base and crosslinker were mixed and degassed until no bubbles were present under vacuum. The sample was then tested in a TA Discovery HR3 Rheometer. The PDMS was initially cured in situ at 60°C for 3 h, using a 40 mm parallel plate, under oscillating time sweep at a frequency of 1Hz and oscillating amplitude of 1 10^{-4} rad. Once the sample had cured it was cooled to room temperature (25°C) and left to

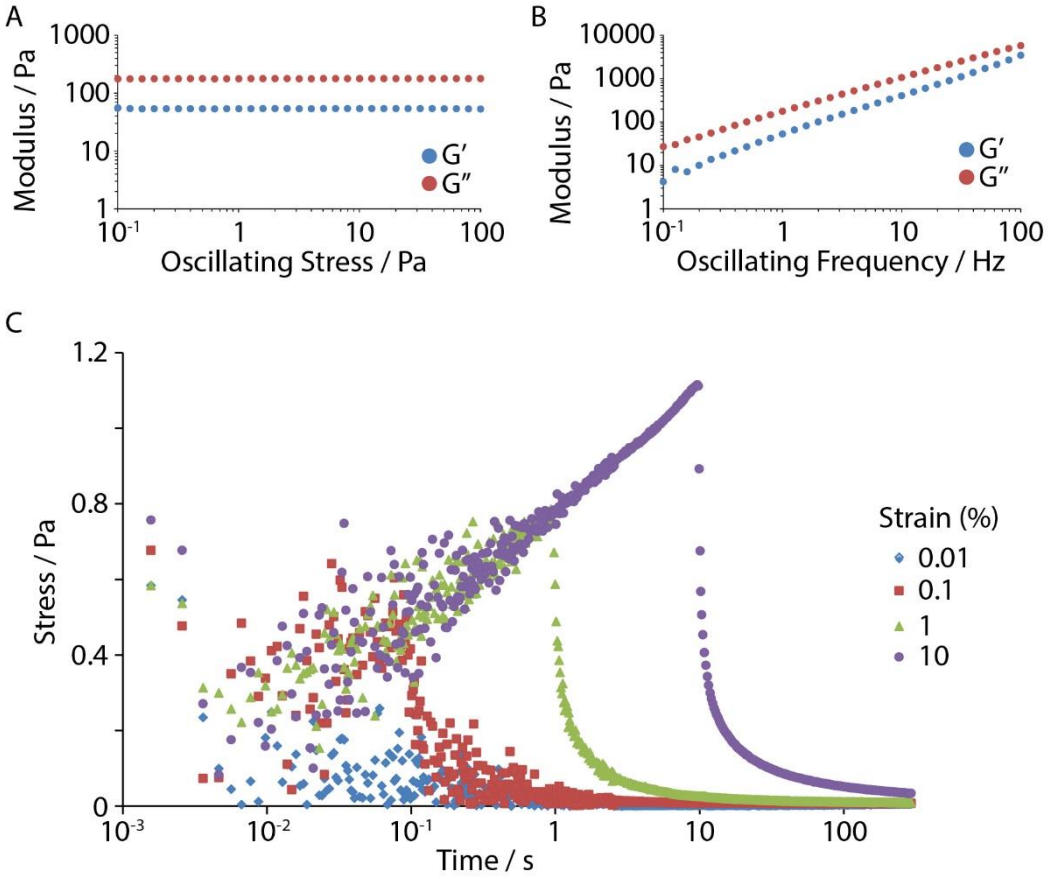

Fig. 1. Mechanical characterisation of Sylgard 184 crosslinked 100:1. Samples were cured in situ and characterised via stress sweep (A, at 1Hz), frequency sweep (B, oscillating amplitude of 10^{-4} rad) and stress relaxation (C, strain rate of 1 %/s).

settle for 300 s before a series of stress relaxation tests were performed. All relaxation tests were 300 s long with a strain rate of 1 %/s and the samples were strained to 0.01 %, 0.1 %, 1 % and 10 %. After the stress relaxation tests, a frequency sweep was performed at a displacement of 10^{-4} rad from 0.1 to 100 Hz on a logarithmic scale getting 10 points per decade. This was followed by a stress sweep performed at 1 Hz, from 0.1 to 100 Pa on a logarithmic scale with 10 points per decade.

7. Interfacial rheology

Rheological measurements were carried out on a hybrid rheometer (DHR-3) from TA Instruments fitted with a double wall ring (DWR) geometry and a Delrin trough with a circular channel. The double wall ring used for this geometry has a radius of 34.5 mm and the thickness of the Platinum–Iridium wire is 1 mm. The diamond-shaped cross-section of the geometry's ring provides the capability to pin directly onto the interface between two liquids and monitors the interface properties without complicated sub-phase correction. 19 mL of the fluorinated oil pre-mixed with surfactant were placed in the Delrin trough and the ring was lowered, ensuring contact with the surface, via an axial force procedure. The measuring position was set 500 µm lower than the contact point of the ring with the oil-phase surface. Thereafter, 15 ml of the PBS buffer were carefully syringed on top of the oil phase. Time sweeps were performed at a constant frequency of 0.1 Hz and a temperature of 25°C, with a displacement of 1.0 10^{-3} rad to follow the formation of the protein layers at the interface. The concentration of BSA used for all rheology experiments was 1 mg/mL (with respect to aqueous phase volume). Before and after each time sweep, frequency sweeps (with a constant displacement of 1.0 10^{-3} rad) were conducted to examine the frequency-dependant characteristics of the interface whilst amplitude sweeps (with constant frequencies of 0.1 Hz) were carried out to ensure that the chosen displacement was within the linear viscoelastic region.

B. Results and Discussion

1. Stress relaxation properties of weakly crosslinked PDMS

Having previously established that human primary keratinocytes and mesenchymal stem cells adhered well even at the surface of ultra-soft PDMS (100:1 crosslinker ratio of Sylgard 184 PDMS), we further examined the mechanical properties of this specific type of materials. In particular, we aimed to determine whether the rate of deformation applied by cells could account for the behaviours observed, as weakly crosslinked viscoelastic materials are known to display strong frequency dependent profiles[28]. We crosslinked Sylgard 184 (base:crosslinker 100:1) in situ in a rheometer and carried out amplitude and frequency sweeps (Figure 1A and B). We

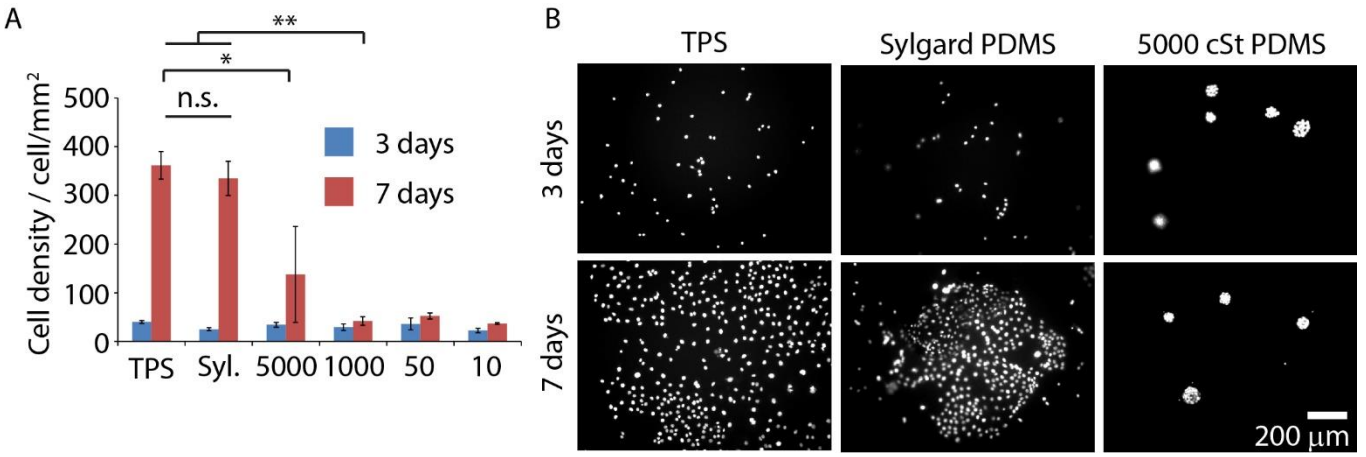

Fig. 2. A. HaCaT cells proliferation on silicone-based liquids. TPS, tissue culture polystyrene; Syl, Sylgard 184; 5000/1000/50/10 describe the viscosity (cSt) of the PDMS oils used. B. Corresponding nuclear staining images.

observed a strong frequency dependent profile (quasi-linear log-log relationship) of the shear moduli as a function of the oscillation frequency (Figure 1B), with the storage modulus varying by nearly 3 orders of magnitude over the frequency range probed. Interestingly, the highest moduli, measured at a frequency of 100 Hz, was only slightly above 3.4 kPa (corresponding to a Young's modulus of 10 kPa). However, corresponding the 2 μm displacement (for the periphery of the 40 mm plate geometry) used for these measurements, this would correspond to a rate of 200 μm deformation per second.

Cells adhere to 2D materials and interfaces via the formation of focal adhesions thought to be the main elements responsible for the transmission of contractile forces to the extra-cellular matrix[29-32]. These adhesions, typically in the range of 0.5-10 μm^2, apply nN forces to the matrix (typically 1-20 nN)[31,33], mediated by actin polymerisation and actin flow at rates of 100-200 nm/s[24]. Such forces are thought to be oriented mainly in the plane of the substrate (although some out-of-plane contribution has been observed)[34]. Considering these deformations and their rates, cells should sense shear moduli corresponding to a range of 1-10 Pa for the weakly crosslinked 100:1 PDMS studied, significantly below the range typically thought to be sensed (for example by cells spreading on hydrogels)[14,24]. Hence our results indicate that the apparent absence of response of cell spreading on weakly crosslinked Sylgard 184 PDMS cannot simply be explained by the strong frequency dependent mechanical behaviour of this material.

However, the modulus of 100:1 PDMS materials also displayed hallmarks of viscous materials, rather than a viscoelastic behaviour, with the loss modulus slightly higher than the storage modulus of the samples tested over the entire frequency range (Figure 1B). In addition, stress relaxation experiments demonstrated full relaxation of these samples, indicating a classical viscous liquid behaviour (Figure 1C). Cell adhesion to viscous fluids with short relaxation times (a few seconds) is surprising as it should not allow ligand and protein clustering for sufficient periods of time to sustain actomyosin-based cell contractility. Although recent reports gave evidence that increased stress relaxation in viscoelastic hydrogels results in ligand clustering and cell spreading[23,35], such process still requires residual stress to persist for prolonged times (min to h). This suggested that bulk mechanical properties are perhaps not representative of nanoscale interfacial mechanical properties of the PDMS substrates we previously tested and led us to ask whether any bulk crosslinking of PDMS substrates was actually required in order to sustain cell spreading, proliferation and to regulate stem cell fate decision.

2. HaCaT cells proliferate on uncrosslinked liquid Sylgard PDMS

In order to test to which extent crosslinking of PDMS was required to sustain cell proliferation, we simply omitted to crosslink the base Sylgard 184 PDMS mixture (no addition of crosslinker) and seeded HaCaT cells onto the resulting oil interfaces. The tissue culture polystyrene (TPS) surface and the PDMS oil-water surface were treated with a collagen solution (20 μg/mL) in order to sustain cell adhesion, but without any chemical treatment to promote coupling to the corresponding interfaces. After 3 and 7 days of culture, we stained cells with the nuclear staining agent Hoechst 33342 and imaged the resulting samples via epifluorescence microscopy to determine any increase in cell density (Figure 2). We clearly observed the formation of cell colonies on TPS as well as Sylgard 184 liquid PDMS, with comparable cell densities 7 days after culture.

Recently, two articles have reported the impact of matrix viscosity and stress relaxation on the clustering of ligand receptors and the ability of cells to spread at the surface of or within the corresponding materials[23,35]. It was proposed that the stress relaxation of viscoelastic materials enables the reorganisation of ligands, upon loading under cell-mediated tension, enabling the clustering of integrins and the generation of cell-mediated traction forces supporting the assembly of the actin cytoskeleton and cell spreading. To test further whether Sylgard PDMS could display sufficiently strong viscoelastic properties (despite full stress relaxation even in lightly cured samples, as discussed above), we cultured cells at the surface of liquid PDMS with defined composition (fully methylated PDMS) and controlled viscosity (up to 5000 cSt, comparable to the

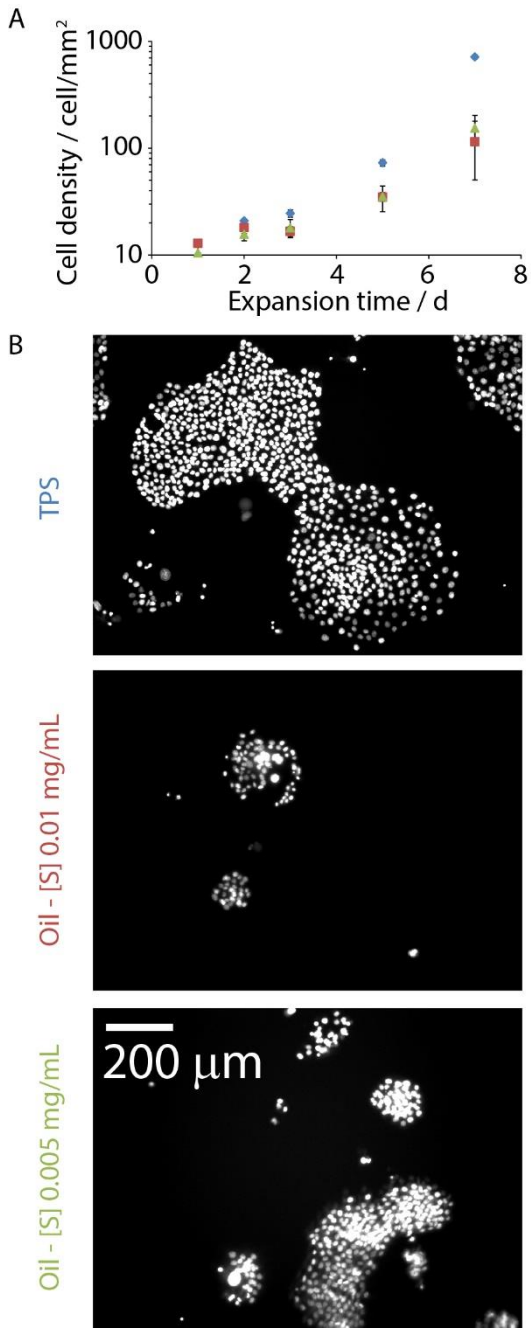

Fig. 3. A. HaCaT cells proliferation profile on interfaces conditioned with cell culture medium (containing 10 % FBS); blue diamonds, TPS; red square, Novec 7500 + 0.01 mg/mL PFBC; green triangles, Novec 7500 + 0.005 mg/mL PFBC). B. Corresponding nuclear staining images (at day 7).

viscosity of Sylgard 184 PDMS. However, no significant cell proliferation was observed (Figure 2), although some small, non-spread colonies were observed in some of the experiments (but not all) carried out on the most viscous PDMS oil tested.

This highlighted that the proliferation of HaCaT cells observed on liquid Sylgard 184 PDMS is not resulting purely from its high viscosity. However, Sylgard PDMS is a relatively complex mixture of alkene functionalised PDMS and alkene-silane

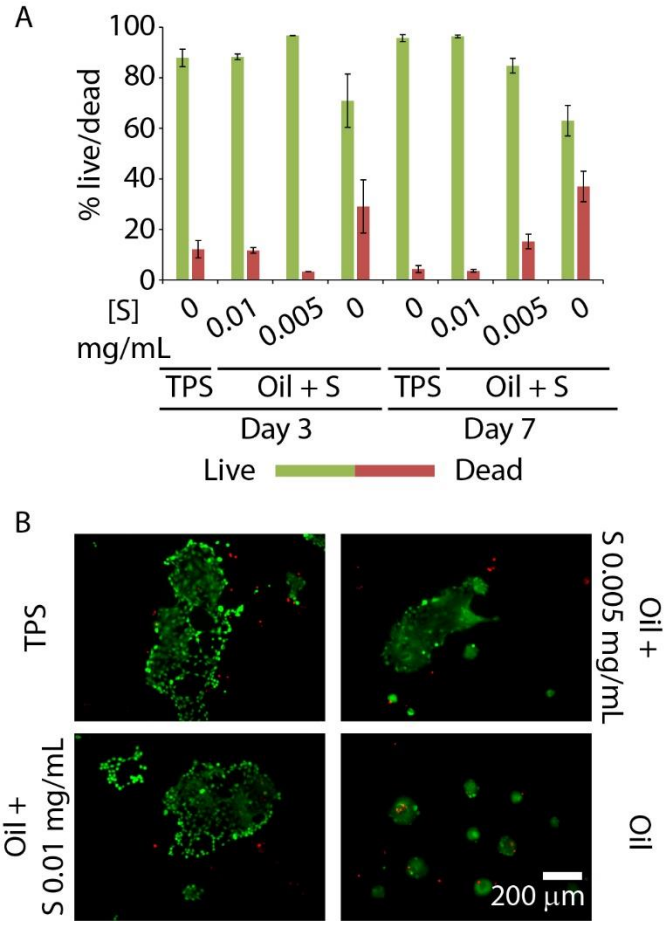

Fig. 4. A. HaCaT cell viability (live, green; dead, red) at days 3 and 7 when cultured on interfaces conditioned with cell culture medium. TPS, tissue culture polystyrene; Novec 7500 + PFBC (0, 0.005 and 0.01 mg/mL). Error bars are s.e.m.; n=3. B. Corresponding images at day 7.

functionalised materials (full composition not disclosed) and our results suggested that perhaps some of the components present in Sylgard PDMS may alter the interfacial properties of the resulting materials or oils, when in contact with cell culture medium. In fact, the culture of cells (fibroblasts) on liquid substrates (PDMS and fluorinated oils with low viscosities) has been reported previously by Keese and Giaever in the 80s[36,37]. In such cases, the authors showed that fibroblasts proliferate at relatively high rates on liquid substrates. They also highlighted that the presence of surfactants in the oil phase was required to mediate such phenomenon[38]. Hence, our experiments and studies indicate that bulk mechanical properties are not necessarily required to mediate cell adhesion and proliferation and that liquids can support such processes

3. HaCaT cells proliferate on a low viscosity fluorinated oil

In order to probe further the phenomenon of cell culture at liquid-liquid interfaces, we repeated the experiment of Keese and Giaever with a fluorinated carrier commonly used in the field of droplet microfluidics, Novec 7500 (viscosity 0.77 cSt). We used a surfactant that was reported to mediate the growth of fibroblasts, pentafluorobenzoyl chloride (PFBC). In the

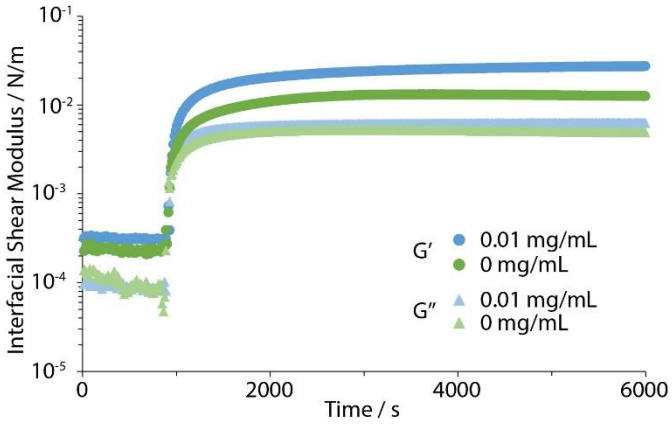

Fig. 5. Time sweep interfacial rheology experiments at interfaces between Novec 7500 oil containing PFBC (0 and 0.01 mg/mL) and PBS containing 10% FBS. 0.1 Hz, oscillating amplitude of 10^{-3} rad.

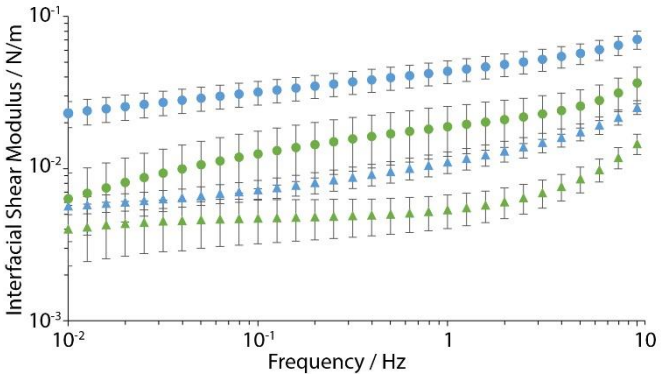

Fig. 6. Oscillatory shear response of interfaces generated from PBS solutions containing 10% 5000 oil containing no PFBC (green) and 0.01 mg/mL PFBC (blue). Circles, G'; triangles, G''. Oscillating amplitude of 10^{-3} rad.

absence of surfactant, no significant cell culture was observed. However when PFBC was added at a concentration of 0.005 or 0.01 mg/mL, significant amounts of cell proliferation was observed, with relatively large and spread colonies apparent after 7 days of culture, although smaller than those formed on tissue culture polystyrene (TPS, Figure 3). This is in good agreement with the reports of Keese and Giaever, which showed that fibroblasts proliferated at high rates, although direct comparisons with tissue culture polystyrene was not made. Our results indicate that such behaviour is also applicable to the case of epithelial cells such as HaCaTs.

We examined whether differences between proliferative behaviours observed on TPS and at oil interfaces were due to toxicity of some of the oil/surfactant mixtures used to generate liquid-liquid interfaces (Figure 4). We observed that cell viability was high (comparable to TPS) when cells were cultured on oil interfaces generated in the presence of PFBC. When no surfactant was used, few rounded colonies formed and displayed increased toxicity, although viabilities remained close to 70%. Hence our viability assay suggests that rather than being due to the toxicity of components of the Novec 7500/PFBC mixtures, the decreased proliferation of HaCaT cells compared to TPS is due to other signals, potentially mechanical.

However, HaCaT cells being adherent cells requiring integrin binding for their proliferation (as for primary keratinocytes)[39,40], the fact that cell proliferation and the formation of spread colonies was possible on liquid substrates suggested that the formation of mechanically strong interface was occurring. This would be likely mediated by the adsorption of proteins from the medium and serum (FBS). Protein adsorption is known to strongly impact cell adhesion and proliferation to solid substrates, including hydrophobic materials[41,42], although it is typically not thought to impact the mechanical properties of the matrix that cells sense when exerting forces to sustain spreading. However, weak anchorage has been reported to result in cell detachment, despite initial adhesion, due to ECM proteins being peeled off the corresponding substrate[43].

Protein adsorption also occurs at liquid-liquid interfaces and is known to mediate important processes such as the stabilisation of emulsions or foams[44]. Hence we proposed that protein adsorption to the PDMS and fluorinated liquid-liquid interfaces studied, modulated by the presence of surfactants or additives, mediated cell adhesion and proliferation.

4. Cell proliferation on fluorinated oil is mediated by a strong mechanical interface

Keese and Giaver also came to the conclusion that protein films must form at liquid-liquid interfaces and be sufficiently mechanically strong to sustain contractile forces generated[45]. Using a modified viscometer, they estimated the mechanical strength of the interfaces generated, in the range of 0.005 to 0.07 N/m and found that fibroblast proliferation reached a plateau at concentrations of PFBC above 10 μg/mL. In their measurements, they specifically investigated the mechanical properties of interfaces generated from albumin solutions, rather than formed in the presence of cell culture medium. To quantify the mechanical properties of the interfaces we generated, we used interfacial shear rheology and a du Noűy ring with a diamond-shape crossection, limiting the shear contribution from the fluid phases[46]. Upon injection of 10% FBS to the aqueous phase (PBS) of an interface formed with the fluorinated oil Novec 7500, the storage shear modulus increased by nearly two orders of magnitude (Figure 5). This increase was relatively fast and complete within 30 min. Similarly, when this experiment was repeated in the presence of 10 μg/mL PFBC, a fast increase of the interfacial storage modulus was observed, with comparable rate, but reaching slightly higher plateau values, above 10^{-2} N/m.

Further investigation of the frequency dependency of the interfacial storage and loss moduli after full equilibration of the protein sheets assembled was carried out (Figure 6). We measured storage moduli significantly higher in the presence of 10 μg/mL PFBC compared to the naked oil (43.6 ± 7.5 mN/m and 18.9 ± 6.8 mN/m, respectively). This is in good agreement with the measurements previously made by Keese and Giaever, who had measured a modulus of c.a. 20 mN/m for an albumin

functionalised interface in the presence of 10 μg/mL PFBC[45], a concentration that was found to support well fibroblast growth and spreading[38]. However, despite the general agreement of the scale of the interfacial moduli measured by Giaever et al. in our study, we found that the interfacial modulus required to sustain cell spreading had to be 2 fold higher (43 mN/m vs 20 mN/m). This could be a result of the greater sensitivity of our experimental set up (weaker contribution of the shear properties of the liquid carriers), and the methodology used (paddle of the viscometer applying a mono-directional deformation in the case of the work of Giaever et al. and oscillating strain in our experiment). These values are also in good agreement with those measured for globular proteins such as lysozyme and albumin associating at oil-water interfaces[47,48]. Hence, we propose that proteins contained in the serum of cell culture medium (such as albumin, which is typically present at mg/mL concentrations in media supplemented with 10% serum) form a stiff sheet at the interface of the two liquids, enabling cell-mediated contractile forces to be sustained.

However, the striking difference observed between cell behaviour on interfaces generated in the absence of PFBC or in the presence of 10 μg/mL of this molecule are surprising as they only arise from a 2-fold increase in the interfacial shear modulus. Interestingly, we observed that the frequency-dependent profile of the two types of interfaces was significantly different (Figure 6). A weaker variation over the range of frequencies probed for interfaces generated was observed in the presence of PFBC. At low frequencies (close to 10^{-2} Hz), the interfacial storage and loss shear moduli measured in the absence of surfactant were found to be very close (only 2-fold higher in the case of the storage modulus), indicating significant changes in the viscoelastic behaviour of these interfaces. Hence it could be proposed that changes in viscoelastic properties regulate cell phenotype at liquid-liquid interfaces, similarly to what was observed for cells interacting at the surface of, or within hydrogels[23,35]. In addition, changes in the type of biomolecules adsorbed at liquid-liquid interfaces, depending on the nature and concentration of surfactants present at corresponding interfaces, are likely to impact cell adhesion and spreading, further contributing to alter cell response at such interfaces.

Conclusions

Overall, our results imply that cell adhesion, proliferation and differentiation at the surface of hydrophobic materials such as PDMS may be regulated by nanoscale mechanical properties of protein interfaces assembled at the surface of such materials, rather than their bulk mechanical properties. We find that the culture of epithelial cells such as HaCaT cells is possible at the surface of very low viscosity liquids, providing a mechanically strong interface is assembled. However several questions remain with such phenomenon. The precise nature of the protein aggregates generated and their interaction with surfactants has not been investigated. The impact of such chemistry on the viscoelastic properties of such interfacial layers could be important and key to the design of a broader range of interfaces supporting the culture of more demanding adherent cell types such as stem cells. In addition, the precise mechanisms via which cell adhesion and phenotype are mediated and regulated at liquid-liquid interfaces are not known and have never been studied: Are they integrin mediated, as in the vast majority of adherent cells spreading on solid 2D surfaces? What are the precise mechanical, morphological and biochemical parameters controlling such interactions? Is stem cell phenotype regulated by such liquid-liquid interfaces? The implications of these questions are high for the design of novel stem cell technologies, for example for the design of emulsion-based 3D bioreactors allowing stem cell expansion or simple delivery.

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References

1 Lutolf, M. P. & Hubbell, J. A. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nat. Biotechnol.* **23**, 47-55 (2005).
2 Discher, D. E., Mooney, D. J. & Zandstra, P. W. Growth factors, matrices, and forces combine and control stem cells. *Science* **324**, 1673-1677 (2009).
3 Guilak, F. *et al.* Control of stem cell fate by physical interactions with the extracellular matrix. *Cell Stem Cell* **5**, 17-26 (2009).
4 Di Cio, S. & Gautrot, J. E. Cell sensing of physical properties at the nanoscale: mechanisms and control of cell adhesion and phenotype. *Acta Biomater* **30**, 26-48 (2016).
5 Dalby, M. J. *et al.* The control of human mesenchymal cell differentiation using nanoscale symmetry and disorder. *Nat. Mater.* **6**, 997-1003 (2007).
6 McMurray, R. J. *et al.* Nanoscale surfaces for the long term maintenance of mesenchymal stem cell phenotype and multipotency. *Nat. Mater.* **10**, 637-644 (2011).
7 Gautrot, J. E. *et al.* The nanoscale geometrical maturation of focal adhesions controls stem cell differentiation and mechanotransduction. *Nano Lett.* **14**, 3945-3952, doi:10.1021/nl501248y (2014).
8 Pelham, R. J. & Wang, Y.-L. Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proc. Natl. Acad. Sci.* **94**, 13661-13665 (1997).
9 Yeung, T. *et al.* Effect of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell Motil. Cytoskeleton* **60**, 24-34 (2005).
10 Peyton, S. R. & Putnam, A. J. Extracellular matrix rigidity governs smooth muscle cell motility in a biphasic fashion. *J. Cell Physiol.* **204**, 198-209 (2005).
11 Tzvetkova-Chevolleau, T. *et al.* The motility of normal and cancer cells in response to the combined influence of the substrate

rigidity and anisotropic microstructure. *Biomaterials* **29**, 1541-1551 (2008).
12 Ulrich, T. A., de Juan Pardo, E. M. & Kumar, S. The mechanical rigidity of the extracellular matrix regulates the struture, motility, and proliferation of glioma cells. *Cancer Res.* **69**, 4167-4174 (2009).
13 Bott, K. *et al.* The effect of matrix characteristics on fibroblasts proliferation in 3D gels. *Biomaterials* **31**, 8454-8464 (2010).
14 Engler, A. J., Sen, S., Sweeney, H. L. & Discher, D. E. Matrix elasticity directs stem cell lineage specification. *Cell* **126**, 677-689 (2006).
15 Saha, K. *et al.* Substrate modulus directs neural stem cell behavior. *Biophys. J.* **95**, 4426-4438 (2008).
16 Gilbert, P. M. *et al.* Substrate elasticity regulates skeletal muscle stem cell self-renewal in culture. *Science* **329**, 1078-1081 (2010).
17 Levental, K. R. *et al.* Matrix crosslinking forces tumor progression by enhancing integrin signalling. *Cell* **139**, 891-906 (2009).
18 Tilghman, R. W. *et al.* Matrix rigidity regulates cancer cell growth and cellular phenotype. *PLoS ONE* **5**, e12905 (2010).
19 Wei, S. C. *et al.* Matrix stiffness drives epithelial-mesenchymal transition and tumour metastasis through a TWIST1-G3BP2 mechanotransduction pathway. *Nat. Cell Biol.* **17**, 678-688 (2015).
20 Trappmann, B. *et al.* Extracellular matrix tethering regulates stem cell fate. *Nat. Mater.* **11**, 642-649 (*2012*).
21 Baker, B. M. *et al.* Cell-mediated fibre recruitment drives extracellular matrix mechanosensing in engineered fibrillar microenvironments. *Nat. Mater.* **14**, 1262-1268 (2015).
22 Huebsch, N. *et al.* Harnessing traction-mediated manipulation of the cell/matrix interface to control stem-cell fate. *Nat. Mater.* **9**, 518-526 (2010).
23 Chaudhuri, O. *et al.* Hydrogels with tunable stress relaxation regulate stem cell fate and activity. *Nat. Mater.* **15**, 326-334 (2016).
24 Moore, S. W., Roca-Cusachs, P. & Sheetz, M. P. Stretchy proteins on stretchy substrates: the important elements of integrin-mediated rigidity sensing. *Cell* **19** (2010).
25 Jiang, G., Huang, A. H., Cai, Y., Tanase, M. & Sheetz, M. P. Rigidity sensing at the leading edge through $\alpha v\beta 3$ integrins and RPTPα. *Biophys. J.* **90**, 1804-1809 (2006).
26 Saez, A., Buguin, A., Silberzan, P. & Ladoux, B. Is the mechanical activity of epithelial cells controlled by deformations or forces? *Biophys. J.* **89**, L52-L54 (2005).
27 Attwood, S. J. *et al.* Adhesive ligand tether length affects the size and length of focal adhesions and influences cell spreading and attachment. *Sci. Rep.* **6**, 34334 (2016).
28 Scanlan, J. C. & Winter, H. H. Composition dependence of the viscoelasticity of end-linked poly(dimethylsiloxane) at the gel point. *Macromolecules* **24**, 47-54 (1991).
29 Galbraith, C. G., Yamada, K. M. & Sheetz, M. P. The relationship between force and focal complex development. *J. Cell Biol.* **159**, 695-705 (2002).
30 Roca-Cusachs, P., Gauthier, N. C., del Rio, A. & Sheetz, M. P. Clustering of $\alpha 5\beta 1$ integrins determines adhesion strength whereas $\alpha v\beta 3$ and talin enable mechanotransduction. *Proc. Natl. Acad. Sci.* **106**, 16245-16250 (2009).
31 Balaban, N. Q. *et al.* Force and focal adhesion assembly: a close relationship studied using elastic micropatterned substrates. *Nat. Cell Biol.* **3**, 466-472 (2001).
32 Geiger, B., Spatz, J. P. & Bershadsky, A. D. Environmental sensing through focal adhesions. *Nat. Rev. Mol. Cell Biol.* **10**, 21-33 (2009).
33 van Hoorn, H. *et al.* The nanoscale architecture of force-bearing focal adhesions. *Nano Lett.* **14**, 4257-4262 (2014).
34 Legant, W. R. *et al.* Multidimensional traction force microscopy reveals out-of-plane rotational moments about focal adhesions. *Proc. Natl. Acad. Sci.* **110**, 881-886 (2013).
35 Chaudhuri, O. *et al.* Substrate stress relaxation regulates cell spreading. *Nat. Commun.* **6**, 6364 (2015).
36 Giaever, I. & Keese, C. R. Behavior of cells at fluid interfaces. *Proc. Natl. Acad. Sci.* **80**, 219-222 (1983).
37 Keese, C. R. & Giaever, I. Cell growth on liquid microcarriers. *Science* **219**, 1448-1449 (1983).
38 Keese, C. R. & Giaever, I. Cell growth on liquid interfaces: Role of surface active compounds. *Proc. Natl. Acad. Sci.* **80**, 5622-5626 (1983).
39 Rahavan, S., Bauer, C., Mundschau, G., Li, Q. & Fuchs, E. Conditional ablation of $\beta 1$ integrin in skin: severe defects in epidermal proliferation, basement membrane formation and hair follicle invagination. *J. Cell Biol.* **150**, 1149-1160 (2000).
40 Boukamp, P. *et al.* Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* **106**, 761-771 (1988).
41 Bathawab, F. *et al.* Lateral chain length in polyalkyl acrylates determines the mobility of fibronectin at the cell/material interface. *Langmuir* **32**, 800-809 (2016).
42 Rico, P. *et al.* Substrate-induced assembly of fibronectin into networks: influence of surface chemistry and effect on osteoblast adhesion. *Tissue Eng. Part A* **15**, 3271-3281 (2009).
43 Guerra, N. B. *et al.* Subtle variations in polymer chemistry modulate substrate stiffness and fibronectin activity. *Soft Matter* **6**, 4748-4755 (2010).
44 Bos, M. A. & van Vliet, T. Interfacial rheological properties of adsorbed protein layers and surfactants: a review. *Adv. Colloid. Interf. Sci.* **91**, 437-471 (2001).
45 Keese, C. R. & Giaever, I. Substrate mechanics and cell spreading. *Exp. Cell Res.* **195**, 528-532 (1991).
46 Fuller, G. G. & Vermant, J. Complex fluid-fluid interfaces: rheology and structure. *Annu. Rev. Chem. Biomol. Eng.* **3**, 519-543 (2012).
47 Freer, E. M., Yim, K. S., Fuller, G. G. & Radke, C. J. Shear and dilatational relaxation mechanisms of globular and flexible proteins at the hexadecane/water interface. *Langmuir* **20**, 10159-10167 (2004).
48 Baldursdottir, S. G., Fullerton, M. S., Nielsen, S. H. & Jorgensen, L. Adsorption of proteins at the oil/water interface - Observation of protein adsorption by interfacial shear stress measurements. *Colloid Surf. B Biointerfaces* **79**, 41-46 (2010).

